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EFFECTS OF HYPOXIA-ISCHEMIA AND ANTI-INFLAMMATORY PROPHYLACTIC
TREATMENT ON CORTICAL AND HIPPOCAMPAL VOLUMES
IN THE DEVELOPING RAT BRAIN

By

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Abstract

Very low body weight as a result of premature birth is a common problem all around the world. Many of these infants have medical issues that arise as a direct result of their very low body weight. One of the biggest issues is a lack of oxygen, which is also known as hypoxia. Hypoxia and ischemia (lack of blood flow) are a problem at any age, but they can be especially devastating to infants who have not undergone critical periods of brain development. Hypoxia-ischemia (or HI) can cause problems that start as inflammation and end with large-scale cell death in the brain (encephalopathy). These pathologies can cause death, or if the infant lives, developmental disorders later in life (Volpe, 2009). Studies done by Lim et al. (2003) have shown promise for the use of inter-alpha-inhibitor proteins (IAIP) to reduce inflammatory damage in a sepsis model. Due to the activity of IAIP as an anti-inflammatory it is possible that it could mitigate the inflammatory damage in the brain caused by neonatal HI. In this study, stereological assessment was performed on 26 male Wistar rats to look for effects of IAIP injected after induction of HI. To measure the effects of the IAIP, volumes of the subjects cortical regions and hippocampal regions were measured after either a sham surgery, HI with an injection of saline or HI with an injection of IAIP. Although statistical analysis of results did not show any significant effect of treatment, this study established a novel paradigm that can be used for this type of research, and gave insight into how future studies could be modified to show a beneficial effect of IAIP injection following HI.

Introduction

Each year in the United States, thousands of infants are affected by hypoxia-ischemia (HI) and as a result, either do not develop properly or die from lack of oxygen. A hypoxic-ischemic incident occurs when a person does not receive adequate blood flow to the brain, and as a result, their brain is deprived of oxygen. In adults this can be caused by a heart attack, stroke or pulmonary embolism and if not treated can cause brain injury or death. In perinatal infants and premature infants, HI insults can cause encephalopathy (disorders and disease of brain tissue) and ultimately death (Volpe, 2009). If the infant does manage to survive the incident they may not develop properly because of the period of time when the brain was starved of oxygen. A combination of detrimental processes occurs during this period of oxygen deprivation which include, but are not limited to generation of free radicals, inflammation and activation of cytokines (cell signaling proteins that can cause inflammation). This cascade leads to apoptosis (or programmed cell death), which emanates from the initial zone of infarct (Allan and Rothwell, 2001). Generally these episodes lead to white matter damage in the brain and are referred to as periventricular leukomalacia (Jantzie, Kathryn and Po-Yin, 2008; Volpe, 2009). A large portion of the infants with PVL go on to have problems with language processing and other cognitive functions. Rates of behavioral deficit can be as high as 25-50% (Volpe, 2009). These issues can range anywhere from spatial memory deficits to rapid auditory processing problems. It is thought that these early deficits in rapid auditory processing could possibly manifest later in life as language comprehension issues. This is thought to occur because the individual can lack the ability to distinguish small changes in rapidly changing auditory signals, which are the basis of language. (Fitch, Threlkeld, McClure and Peiffer, 2008; McClure, Threlkeld and Fitch, 2007)

As with all diseases and disorders, identifying the factors involved in pathogenesis could provide targets for therapeutic intervention. Over the past year, research was done at Rhode Island College to investigate possible therapeutic courses for this injury profile. The overall goal of the lab is to find a way to effectively treat or prevent the brain damage resulting from these hypoxic ischemic episodes as well as treating any long-term behavioral or learning impairments using a neonatal rat model of hypoxia-ischemia (HI). In the current line of work, immunomodulator inter-alpha-inhibitor protein (IAIP) was investigated as a potential treatment for neonatal HI injury. These IAIP have been shown to limit the harmful inflammatory effects of cytokines, inhibit destructive proteases and treat sepsis, among other neuroprotective actions in adult rodent models (Allan et al., 2001; Singh, Zhang, Bendelia, Heath, Murphy, Sharma and Lim, 2010). The roles of IAIPs are not yet fully understood, but it is known is that they are proteins found in the serum of mammals consisting of a light chain connected to two heavy chains (Zhuo, Hascall and Kimata, 2004). Studies have been done on the sepsis model that show that as the injury pathology progresses the levels of IAIP in the blood drop in accordance with the course of the injury. This observation led to the hypothesis that they played a role in mediating the kind of damage resulting from these septic inflammatory injuries (Lim, Bendelja, Opal, Siryaporn Hixson and Palardy, 2003). Studies have shown positive effects of injecting mice with IAIP after an injection of *E. coli* (a bacterium that causes sepsis). An injection of 30mg/kg body weight of IAIP administered 45-60 minutes after the injection of *E. coli* increased the 50% survival dose 100 times (Lim et al., 2003). Since IAIP showed a beneficial role in mediating inflammatory response resulting from sepsis, it could also be possible that IAIP could show a positive effect in mediating the inflammatory response resulting from a hypoxic-ischemic injury.

When looking at the inflammation and cell death resulting from hypoxia-ischemia, two particular areas of interest in the brain are the cortex and the hippocampus. The cortex is the outer layer that surrounds the rest of the brain and has a wide array of functions. One that can be seen across species is control of motor function. The motor cortex of an organism is mapped out such that every body part has a corresponding section of motor cortex to control movement. Body parts that require finer control are represented by a proportionately larger section of motor cortex (Hosp and Luft, 2011). The cortex doesn't just play a role in sensory or motor processing, its functions reach as far as mediation of other brain regions. The amygdala serves to mediate certain emotional responses, but the cortex is able to override these impulses to alter behavior (Stephens and Duka, 2008). The prefrontal cortex has also been shown to play an integral role in working memory in humans (Courtney, Petit, Maisog, Ungerleider and Haxby, 1998). In a study preformed by Wishaw et al., it was shown that rats with damage to the cingulate of the cortex lost the ability to navigate a water maze. The results of this study supported the idea that the affected parts of the cortex had an important role in generating behaviors that depended on the associations of stimuli, events and cues (Sutherland, R. J., Wishaw, I. Q., and Kolb, B., 1988). The hippocampus has been shown to play a role in memory and learning. Damage to specific portions of the hippocampus has been shown to cause reduction in spatial learning abilities and visual recognition (Morris, 1984). The hippocampus has also been shown to play an important role in episodic learning (Treves and Rolls, 1994). While hypoxic ischemic events could produce different pathologies for hippocampal damage, anytime the hippocampus is damaged a deficit in some aspect of memory will follow (Treves et al., 1994). In a study performed by Auer et al. (1989), deficits to learning and memory were seen after lesioning of the CA1 field of the hippocampus. This study showed the involvement of the hippocampus because they were able to

limit the damaged area of the brain to the hippocampus in order to rule out deficit due to damage to other areas (Auer, Jensen and Wishaw, 1989).

A number of studies on this subject have already been done, and continuing research in this field may overlap with research on hypoxia-ischemia or other disorders affecting adults. Studies have looked at the physiology of a developing brain and specifically how the oxygen deprivation affects it (specifically which structures of the brain are effected) as well as how these effected regions can impact the development of a child (what roles do these damaged regions play in cognitive function). One study in particular looks at the time between birth and hypoxic-injury in rats and looks at how the timing of the injury affects the development process (McClure et al., 2006). Knowing how HI insults occurring at different times after birth effect patients differently could lead to more effective and targeted options for treatment. We hypothesized that IAIP treatment following neonatal HI will lead to improved outcome as evidenced by a reduction in neuronal cell death.

The primary aim of the project was to evaluate the early neuroprotective effects of systemic inter-alpha-inhibitor protein administration on brain injury in the cortex and hippocampus of rats that underwent hypoxia-ischemia, more specifically, looking at cortical and hippocampal volumes in post natal day 7 rats receiving IAIP injections 72 after HI. Currently there is a lack of extensive research in the neonatal rat model evaluating the effects IAIPs on early neuronal injury. The research was performed on rats because neonatal HI in this species mimics the human pathological cascade and anatomical outcome, and the rats were at P7 because of the resemblance in brain structure and development of a P7 rat and a term human infant (McClure et al., 2007).

Methods

Subjects and Surgery

Subjects used were male Wistar rats born at Rhode Island College from dams purchased and shipped from Charles River Laboratories (Wilmington, MA). Dams were kept in individual housing until the pups were born. Pups were divided among the mothers into groups of 8 males and 2 females (to adjust gender ratios). All subjects were kept on a 12 hour light/dark cycle and had food and water available *ad libitum*. On postnatal day 7 the pups were randomly divided into 3 groups: sham, HI with vehicle (saline) treatment and HI with IAIP treatment. The pups were anesthetized with isoflurane and an incision was made midline on the neck running vertically. Each sham pup was then sutured and placed on a heating pad. Both of the HI groups had the right common carotid artery cauterized before being sutured. All pups received ink injections into their paws for identification purposes. Throughout the course of the surgery pups were kept warm with heating pads to maintain a normal body temperature and to avoid any neuroprotective effects of hypothermia (Higgins, Raju, Edwards, Azzopardi, Bose, Clark, Ferriero, Guillet, Gunn, Hagberg, Hirtz, Inder, Jacobs, Jenkins, Juul, Laptook, Lucey, Maze, Palmer, Papile, Pfister, Robertson, Rutherford, Shankaran, Silverstein, Soll, Thoresen and Walsh, 2011). Pups were allowed to recover for a period of 1-3 hours before the HI-vehicle group received an injection of NaCl solution and the treatment group received injections of IAIP. All injections of IAIP proteins were given based on the bodyweight of the subjects with doses at 30mg/kg. Injections were given intraperitoneally. All pups were then placed in a reduced oxygen environment (8% balanced with 92% nitrogen) for 90 minutes to provide a hypoxic environment. Treatment subjects also received another injection of IAIP or saline 24 hours post HI induction. Rats were then returned to their mothers to recover. All rats were maintained in clean housing

with the same 12 light/dark cycle and food and water supply previously mentioned (McClure, 2007). All procedures were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, including adequate measures to minimize pain and discomfort. The Institutional Animal Care and Use Committee (IACUC) at Rhode Island College approved all procedures.

Histology

On P 10 subjects were sacrificed using pentobarbital at a dose of 100mg/kg and the brains were fixed (4% paraformaldehyde) and embedded in paraffin. Brain samples were sent to Rhode Island Hospital Core Research Services to be mounted on slides for staining. Every 20th section of brain was sectioned and mounted in the coronal plane on glass slides. The slides were sectioned from anterior to posterior. Once the samples were returned they were stained with a thionine staining protocol. Slides went through a series of alcohol gradients (100%,100%, 95%,70%,50%,distilled water,distilled water) in order to rehydrate the samples. At this time the sample were placed in a 1% thionine solution for 15-18seconds to be stained. After staining the slides went through another alcohol gradient (distilled water, distilled water, 50%,70%,95% with acetic acid, 95%, 100%, 100%) to dehydrate the samples. Samples then went through two Citrisolv baths before they were coverslipped with DPX mounting solution. Slides were left to dry overnight.

Image analysis

Following staining, images of each sample were taken using a Nikon Eclipse E600 microscope, using the program QCapture. The images were then analyzed using ImageJ software. The scale was set so that 266 pixels on screen corresponded to 1mm (Figure 1). Images were overlaid with a grid of 0.02mm box size that was randomly overlaid on each image. While counting the images, the experimenter was unaware which treatment group the images came from. With the 0.02mm grid size the grid intersections falling on the cortex of each image were counted. Counting started at the top of the section and went down to the rhinal fissure. The images which contained the hippocampus were then overlaid with a grid with 0.01mm boxes and the grid points falling on the hippocampus were all counted. All data was recorded and entered into a Microsoft Excel spreadsheet for analysis. The data was then analyzed using PASW statistical software where a two-tailed analysis of variance (ANOVA) was performed.

Results

In figure 1 a prototypical example of a sham subject (top), an HI subject (middle) and an IAIP subject (bottom) can be seen. The dense purple regions correspond to higher neuronal density. The neuronal density in the right cortex of the middle section is noticeably lower than the top or bottom sections.

A two way ANOVA test across three treatment groups (sham n=8, HI n=10 and IAIP n=8) revealed no significant difference between right cortical volumes of subjects ($F=(2, 23) = 1.16, p = 0.331$) (Figure 2). The two way ANOVA test also showed no significant effect of treatment on right hippocampal volume ($F=(2, 23) = .967, p = 0.388$) (Figure 3). In both cases however, there was a trend towards lower brain volume in both the HI and IAIP groups.

Discussion

In this study, treatment of P7 neonatal rats with IAIP, post hypoxia-ischemia and again 24 hours later, did not yield any statistically significant effects on the volumes of the ipsilateral cortex or hippocampus. Contrary to the literature we did not see a beneficial effect of IAIP injections on gross brain volume (Lim et al., 2003). The results we observed could be explained in a number of ways. First, the sample size (N=26) may not have been large enough. Although there was a trend of the sham subjects having larger brain volumes than the HI or the IAIP groups, there was no significant effect. The sham group served as a control because it is known that a hypoxic-ischemic event causes brain damage and a reduction in brain volume (Volpe, 2009), so the fact that there was no significant difference between the sham group and HI group could mean that a larger sample size would be needed to increase statistical power. One factor which could have led to the unexpected results could have been the histology process. After the brains were sent to Rhode Island Hospital to be sectioned and mounted there were noticeable cracks, tears and some folds on the tissue. This could have been caused from expansion and contraction when mounting or knife artifacting when cutting. Although the quality of the tissue did improve with each subsequent set sent for processing, discrepancies in data from earlier samples could have skewed the data when the total sample size is taken into consideration. In future experiments if all samples are processed in a manner that results in less damage the variability between the brain volumes could be less, as the standard deviations within groups for volumes of structures are high. The group with the highest variability was the HI group. This could be in part due to the inherent variation between injury profiles between subjects, or the degree to which they were damaged during processing could have been the highest since they

were the group with the largest sample size (n=10 compared to n=8 for both the sham and IAIP groups).

Moving away from the possible problems with the preparation of the tissue samples, in the future if the experiment were to be repeated the amount of IAIP could be altered. Although the amount given in this experiment (30mg/kg) is the same as the amount given in sepsis studies, and in those studies it showed a significant effect (Lim et al., 2003), the injury profiles for sepsis and HI are discrete from one another and as a result the treatment dosage may need to be altered. Since this was only the first experiment of its kind the effects of a larger dose, possibly 60mg/kg, could show a significant effect on the cortical and hippocampal volumes. Finally it is a possibility that the treatment had a significant effect on a different measure of brain damage which was not examined in this study. Analysis of other brain regions (such as the corpus callosum) may show a statistically significant effect. The type of measure we used (gross brain volume) may not be the most sensitive measure for this kind of injury profile, so different measurement techniques may need to be used to assess the effectiveness of IAIP. One possibility is to count neuronal death using a fluorescent dye as opposed to using a thionine stain to measure volume. Although the volume of the subjects brains may not have been changed significantly in this experiment the density of neurons, or their organization may have changed. To further explore this possibility, behavioral testing should be done on subjects to look for any significant effects on motor function, working memory and spatial navigation. Finally, it is possible that the subjects were sacrificed too early (P10). If the subjects were allowed to mature more and brain development was to continue, an effect on brain volume may have been observed.

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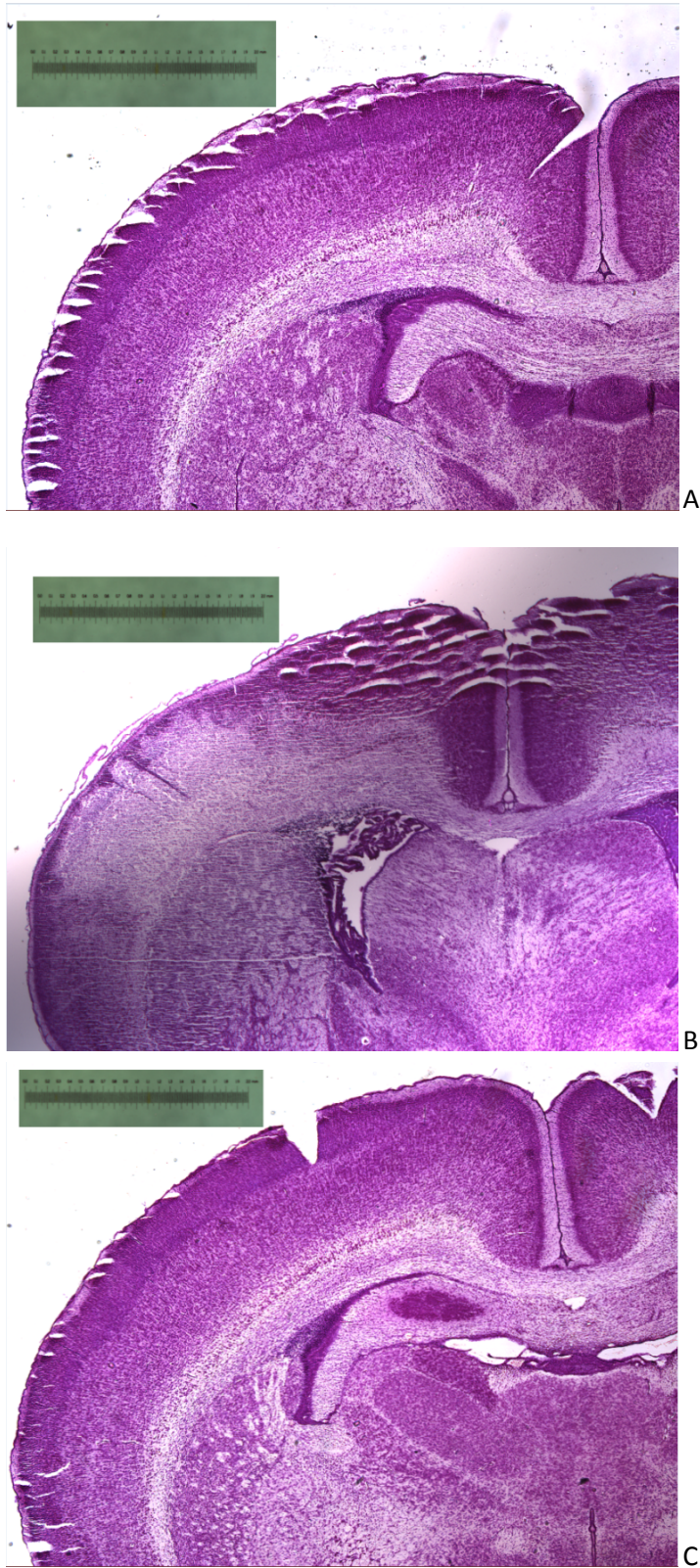


Figure 1. 140th section of a sham subject (A). 140th section of an HI subject (B). 120th section of an IAIP subject (C). All images of upper right quadrant. All scale bars set to 2.0mm.

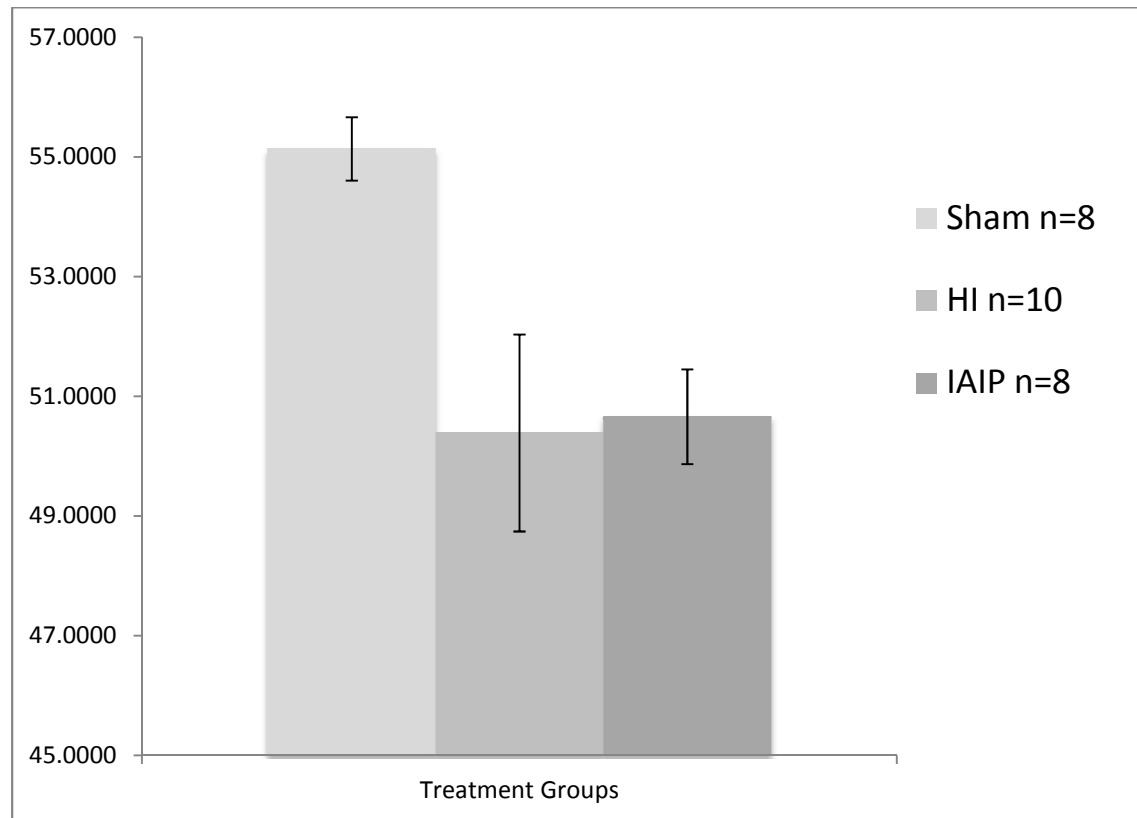


Figure 2. Cortical volumes (cubic millimeters) of treatment groups. Sham mean= 55.1335625, SEM= +/- 1.05727257533197. HI mean= 50.38715, SEM= +/- 3.2924508391251. IAIP mean= 50.6581875, SEM= +/- 1.583486591404.

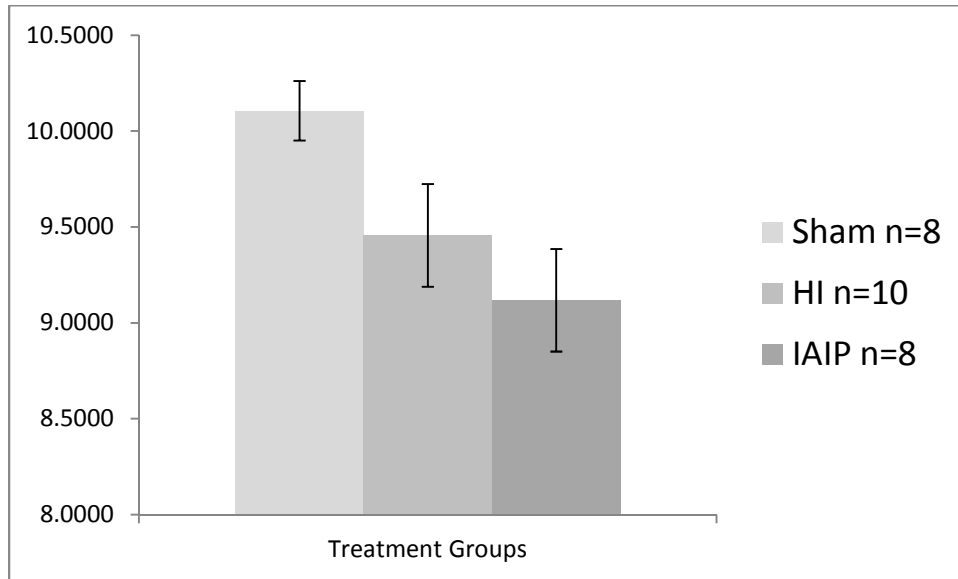


Figure 3. Hippocampal volume (cubic millimeters) of treatment groups. Sham mean= 10.10590625, SEM= +/- 0.309317570201712. HI mean= 9.4566, SEM= +/- 0.535542540586439. IAIP mean= 9.118, SEM= +/- 0.53523179641428.